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Incorporation of water-in-oil-in-water ($W_1/O/W_2$) double emulsion in a set-type yogurt model

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Abstract

The effect of $W_1/O/W_2$ emulsion incorporation in set-type yogurt on the acidification process, physicochemical properties, bacterial growth kinetics and structural characteristics was investigated. The $W_1/O/W_2$ emulsion was formed by using a two-step homogenisation process and milk as the W_1 and W_2 phases, and stability was monitored with optical microscopy and cryo-SEM. Adding the $W_1/O/W_2$ emulsions reduced the acidification rate, viscosity and water retention capacity. Texture (adhesiveness, cohesiveness, hardness, and gumminess) differed in yogurts containing $W_1/O/W_2$ emulsion compared to controls during the acidification process, however, trends became stable during storage. The growth of *S. thermophilus* during the acidification process of yogurt was reduced in the presence of $W_1/O/W_2$ emulsion while *L. bulgaricus* trended higher during storage. This study shows that yogurts containing $W_1/O/W_2$ emulsion are feasible subject to processing

modification.

Keywords: yogurt; $W_1/O/W_2$ double emulsion; acidification; *Streptococcus thermophilus*; *Lactobacillus bulgaricus*

1. Introduction

Water-in-oil-in-water ($W_1/O/W_2$) emulsions are common type of double emulsions (DE) that are formed by dispersing a water-in-oil (W_1/O) in a continuous aqueous phase (W_2). $W_1/O/W_2$ emulsions can facilitate encapsulation in foods, cosmetics and drug delivery. In foods, $W_1/O/W_2$ emulsions can be used to make low calorie products, control flavour release, salt reduction and food fortification by encapsulating vitamins, minerals, anti-microbial agents, antioxidants, and amino acids, (Muschiolik and Dickinson, 2017).

In fermented foods, the introduction of $W_1/O/W_2$ emulsions assume further complexity due to interface-bacteria interaction in the continuous phase and/or bacteria encapsulated in the inner phase. Experimentally, $W_1/O/W_2$ emulsions were introduced in cheese (Lobato-Calleros, Rodriguez, Sandoval-Castilla, Vernon-Carter & Alvarez-Ramirez, 2006); Lobato-Calleros, Sosa-Pérez, Rodríguez-Tafoya, Sandoval-Castilla, Pérez-Alonso & Vernon-Carter, 2008) and more recently in a stirred-type yogurt for the encapsulation of caffeine (Hernandez-Marín, Lobato-Calleros, Roman-Guerrero, Alvarez-Ramirez, & Vernon-Carter, 2016).

Research on microbial encapsulation in $W_1/O/W_2$ emulsion has been limited to the encapsulation and protection of probiotics, for example, *Lactobacillus* spp. were protected in the inner phase of $W_1/O/W_2$ emulsion from cytotoxic gastric juice models and bile salts (Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas & Vernon-Carter, 2009; Shima, Morita, Yamashita & Adachi, 2006; Shima, Matsuo, Yamashita & Adachi, 2009).

Yogurt is a fermented dairy product associated with nutrient fortification e.g. probiotics. Set-type yogurt has a characteristic thick texture and is a natural emulsion produced by milk acidification into containers without any further stirring, i.e. the fermentation of lactose to lactic acid by suitable bacterial cultures which decreases the pH. As pH decreases, caseins form aggregates initiating the formation of protein clusters which are connected by thin strands leaving serum captivities (Tamime and Robinson, 2007). For set-type yogurt, a firm gel with smooth homogeneous consistency, and no expelled whey at the surface are the desired textural properties (Tamime et al., 2007). The presence of $W_1/O/W_2$ emulsion, during acidification could alter the physicochemical characteristics and the stability of the yogurt structure (Hernandez-Marín et al., 2016). The presence of bacteria within the continuous phase in yogurt can alter the stability of emulsions depending on bacterial characteristics such as metabolic activity, surface charge and hydrophobicity (Li, Jiang, Liu, Chai, Li, Li, & Leng, 2012; Dorobantu, Yeung, Foght, & Gray, 2004; Ly, Naïtali-Bouchez, Meylheuc, Bellon-Fontaine, Le, Belin & Wache, 2006; Boitard et al., 2012; Firoozmand & Rousseau, 2014). Bacterial cells with opposite surface charge to the emulsion droplet will destabilise the emulsion structure by aggregating around the oil globules (Ly et al., 2006). Furthermore, the emulsion can affect bacterial growth rate as well as the form of growth (planktonic versus colony formation). The presence of emulsion limits the diffusion rate of nutrients and cause a reduction in the growth rates of bacteria (Brocklehurst, Parker, Gunning, Coleman & Robins, 1995) and increase in oil phase concentration of emulsions constrained bacteria to grow as colonies opposed to growing in planktonic form

(Brocklehurst et al., 1995).

The potential of $W_1/O/W_2$ emulsions in fermented food would expand by controlling the release of encapsulates and bacteria. So far controlled release in $W_1/O/W_2$ emulsions without bacteria was demonstrated based on tailoring the osmotic pressure of the water phase or pH triggered release with pH-responsive emulsifiers. Changes of pH and osmotic gradients are common in food fermentation and digestion, therefore, there is fascinating potential for $W_1/O/W_2$ emulsions to encapsulate species as well as controlling release in response to pH/osmotic changes in formulation during production and post-consumption. Developments on pH-responsive release of $W_1/O/W_2$ emulsions include natural, food grade biopolymers such as xanthan gum (XG) can interact non-covalently with other components forming colloidal coacervates with pH responsive properties (Patel, Drost, Seijen ten Hoorn & Velikov, 2013).

In the case of osmotic pressure-based destabilisation of $W_1/O/W_2$ emulsions, polysaccharides or salts were dissolved and the size of the inner droplets was manipulated by altering the chemical potential of the external and internal water phases and resulting to water transport and swelling/shrinking (Mezzenga, Folmer, & Hughes, 2004). Recently, the authors investigated the release mechanism of GFP-tagged *Escherichia coli* in a $W_1/O/W_2$ model by altering the osmotic balance, through increasing salt concentration, as it could take place in fermentation (El Kadri, Overton, Bakalis & Gkatzionis, 2015; El Kadri, Gun, Overton, Bakalis & Gkatzionis, 2016). Bacterial release was found to be affected by the concentration of emulsifiers, osmotic balance alteration

and, interestingly, during osmotic imbalance at low concentration of surfactants, the release of *E. coli*-GFP was significantly affected by volume of W_1 and release occurred due to the bursting of the oil globules independent to diffusion mechanisms.

Encapsulation of microbes in dairy has been previously achieved using polymers (e.g. alginate) (Sultana et al., 2000; Krasaekoopt et al., 2006). However, the use of $W_1/O/W_2$ emulsion is more suitable as it can be made from dairy ingredients or it can be used to replace animal fat with healthier oils since studies have shown that substitution of dairy fat with vegetable sources reduces the risk of cardiovascular disease (Chen et al., 2016; Ryeo-Eun et al., 2015). Therefore, the ultimate aim is to develop a yogurt system containing $W_1/O/W_2$ emulsion that facilitates the encapsulation of multiple bacterial species in the inner W_1 phase to deliver dietary benefits and to achieve the fermentation within the continuous W_2 phase. However, before $W_1/O/W_2$ emulsions can be used for encapsulation and controlled delivery of microbes it is important to understand their effect on food structure, physicochemical properties, and sensory characteristics and their stability during manufacturing and storage.

The main objective of this work was to investigate the feasibility of incorporating $W_1/O/W_2$ emulsions within set-style yogurt and potential application to the dairy industry. In the present study, the effect of a model $W_1/O/W_2$ emulsion on a set-style yogurt was investigated during the fermentation process in terms of acidification profile, bacterial growth, and physicochemical properties. By using milk as W_1 and W_2 phases, the O-W_2

interface was stabilised solely by the milk proteins without using synthetic hydrophilic surfactants, in order the system to remain food grade. The stability over long term storage (28 days) at 4^{o}C was further investigated by monitoring bacterial survival, physicochemical and textural properties.

2. Materials and Methods

2.1 Materials and microbial cultures

Fresh whole milk, butter and food grade sunflower oil were purchased from a local retailer (United Kingdom). The oil soluble emulsifier polyglycerol polyricinoleate (PGPR) was provided by Danisco (Denmark). Skimmed milk powder, MRS broth, MRS agar ad M17 were purchased from Fisher Scientific (United Kingdom).

A commercially available yogurt starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* at ratio 1:1*)* was purchased from Micromilk (Cremosano, Italy). Microbial Inoculum was prepared by transferring aseptically 0.01 g of the freeze-dried yogurt starter culture in 150ml of sterile reconstituted skim milk (RSM) 10% w/v. The cultures were grown anaerobically at 37°C overnight to ~10^9 colony forming units (CFU)/ml.

Butterfat oil was isolated from butter according to Council Regulation (EC) No 1255/1999 (EC, 1999). Briefly, a butter bar was melted in the oven at 50^o C and the oil phase was purified through Whatman filter paper No1 (11µm) in the presence of anhydrous Na_2SO_4 at 50^oC.

2.2 Preparation of $W_1/O/W_2$ emulsions

$W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at 25^oC. A two-step emulsification process was followed as described by El Kadri et al. (2015) with slight modification. Briefly, in the first step primary W_1/O (milk in oil) emulsions were made. An oil phase was prepared by dissolving 2 % w/w PGPR in sunflower or butterfat oil. In some

experiments, the oil phase was stained using Sudan Red (0.08% w/v) in order to monitor the distribution of the double emulsion within the yogurt. The inner aqueous phase (W1) consisting of milk was emulsified into the oil phase (W_1:O phase ratio of 40:60) at 4000 rpm for 120 sec at 25^{o}C. In the second step $W_1/O/W_2$ emulsion was made using milk as (W_2). The previously prepared primaryW_1/O emulsion was emulsified into W_2 to form the $W_1/O/W_2$ emulsion (W_1/O:W_2 ratio of 20:80) at 2700 rpm for 60 sec.

2.3 Preparation of set-style yogurt models with and without double emulsion

Fortified milk for yogurt production was prepared by adjusting the total soluble solid content to 16% w/v with the addition of skimmed milk powder. Aliquots of 150 ml were transferred in sterile duran bottles (250mL) with screw caps and were pasteurized at 80^{o}C for 30 min.

Fermentation was carried out by inoculating the substrates with 6 % w/v of activated starter culture to a final concentration of ~8.4 cfu/ml, in a water bath at 42^{o}C and pH was monitored every 15 min as described by Lazaridou, Serafeimidou, Biliaderis, Moschakis and Tzanetakis (2014). This inoculum size showed optimal acidification rates (V_{max}) after a preliminary screening test with different inoculum sizes (3%, 6%, 8% and 10%) (data not shown).

The double emulsion composed 33% v/v of the final product. Specifically, 50 mL of double emulsion were introduced in 100 mL of fermenting milk at pH 5.7 ± 0.1 at 180min after initiation of acidification. The samples were mixed gently and left to stand until the pH reached 4.6 ± 0.05. On completion of acidification, the samples were cooled by immersing in ice water and were

stored at 4^{o}C for 24h (day 0).

2.4 Monitoring acidification and microbial growth kinetics during fermentation and storage

The acidification rate (Vmax) was calculated as the time variation of pH (dpH/dt). At the end of each fermentation, kinetic parameters were calculated according to Mishra and Mishra (2013): (1) time to reached Vmax (h), t_{Vmax}; (2) pH at Vmax, pH_{Vmax}; (3) time to complete the fermentation (h)$t_{pH4.6}$.

During acidification, a bottle was withdrawn every 1 h and analyzed for cell growth and selected physicochemical characteristics. During post acidification storage, samples were analyzed at 0, 2, 4, 6, 8, 10, 12, 14, 21 and 28 days.

For bacterial enumeration yogurt samples (1 g) were collected aseptically, serially diluted in phosphate buffered saline (PBS) and were analysed by culture on media using the Miles and Misra technique (Miles & Misra, 1938). Enumeration of *L. bulgaricus* and *S. thermophilus* was conducted on MRS and M17 agar media, respectively, after incubated aerobically at 37 °C for 48 h (Kristo *et al*., 2003).

2.5 Structure characterization of $W_1/O/W_2$ emulsions and yogurt with optical and electron microscopy

Yogurt samples with or without $W_1/O/W_2$ emulsions were placed on microscope slides and images were acquired under 10x magnification using optical microscopy (Zeiss Axioplan) at 25^{o}C coupled with a digital colour camera system (10 megapixel Motic Moticam CMOC camera) via Motic Images Plus video acquisition software.

The microstructure of yogurts samples containing $W_1/O/W_2$ emulsions was visualised using cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESSEM). One drop of each sample was frozen to -180^{o}C in liquid nitrogen slush. Fractuation and etching of the frozen sample was performed for 5 min at -195^{o}C in a preparation chamber. Subsequently, samples were sputter coated with gold and scanned at -160^{o}C.

2.6 Measurement of oil globule size of double $W_1/O/W_2$ emulsions.

The particle size distribution of W_1 droplets in W_1/O and oil globules in the $W_1/O/W_2$ emulsion was measured immediately after preparation using a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd, Worcestershire, UK), equipped with a He-Ne laser (λ = 633 nm). The dispersion unit stirring speed was kept at 2000 rpm and the measurement range was 0.02–2000 μm. The optical parameters selected were: dispersed phase refractive index of n_D^{22} 1.39; oil globule absorbance of 0.01; and a dispersant liquid (distilled water) refractive index n_D^{22} 1.33; obscuration between 10% and 20%. Particle size calculations were based on the MieScattering theory and the volume mean diameter values (D [4, 3]), and the percentage of volume corresponding to each observed population were calculated using the Mastersizer 2000 software.

2.7 Determination of physicochemical properties

Total acidity: Samples of yogurt (9 g) were diluted in 18mL of water and titrated using 0.1 N NaOH and phenolphthalein solution (1% w/v) as an indicator. Total acidity was calculated using the formula <1 mL 0.1 NaOH = 0.009 g lactic acid> and expressed as % w/w lactic acid.

Water retention capacity: 10g of sample were transferred in a plastic conical tube (15mL) and centrifuged at 20000g for 10 minutes. The supernatant was discarded and the water retention capacity was calculated as % w/w of the sediment over the initial weight of the sample.

Syneresis: 5 g of samples were weighted on Whatman filter paper No1 (11 μm) and were drained under vacuum for 10min. Syneresis was expressed as % w/w of the drained liquid over the initial weight of the sample.

Viscosity measurements: Rheological characterisation of the yogurt samples with and without $W_1/O/W_2$ emulsions during and after acidification was performed at 4^{o}C using AR-G2 rheometer (TA instruments, New Castle, Delaware USA) equipped with a 14mm vane spindle. Viscosity of a representative yogurt sample (~30mL) was measured over a shear rate 0-100 s^{-1}.

Texture Analysis: Samples of yogurt (30ml) were distributed to cylindrical plastic vessels (d 140mm) immediately after preparation (day 0) and left to set for another 24h at 4^{o}C.Texture profile analysis (TPA) of the samples was conducted using a Texture Analyzer TAXT2i (Stable Micro Systems, Surrey, England) with accompanying computer software (Exponent). Samples were compressed under a 40mm cylindrical probe (P/40) at a test speed of 1 mm/s and a trigger force of 1 g, using the Texture Analyzer. Two compression cycles at 50% of the initial height were applied using a post-test speed of 4 mm/s. The data obtained from the force–time curves were used to calculate the hardness (g), cohesiveness, adhesiveness (g*s) and gumminess (g). Three replicates per sample were tested.

2.8 Statistical Analysis

Two independent experiments were carried out in all cases and at least three replicate measurements were carried out for each sample. Statistical comparison of the mean values was performed by student's *t*-test ($P<0.05$ confidence level) using the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Error bars represent the standard deviation (sd) of the mean value.

3 Results

3.1 Characterisation of primary W_1/O and $W_1/O/W_2$ emulsions

The size of the W_1 droplets and the oil globules can influence the stability of $W_1/O/W_2$ emulsions (Weiss & Muschiolik, 2007). The homogenisation conditions in this study (section 2.2) were such that the average mean size distribution [D (4, 3)] of W_1 droplets and oil globules were comparable (10-15 µm and 70-80 µm, respectively) in all the emulsions (Fig. S1).

3.2 Stability of $W_1/O/W_2$ emulsions in yogurt

Two $W_1/O/W_2$ emulsion systems made with different dispersed phases (butterfat or sunflower oil) were tested for their effect on yogurt formation and its stability during storage. Butterfat was used due to being natural to yogurt while sunflower oil was tested for vegetable oils used as a healthier substitute of milk fat in set type yogurts (Farmani, Edalatkhah, Motamedzadegan & Mardani, 2016). The microstructure of yogurt with $W_1/O/W_2$ emulsion was observed using optical microscopy. Immediately after the acidification process the $W_1/O/W_2$ emulsion globules made with butterfat were dispersed homogeneously throughout the yogurt, however, flocculation and partial coalescence between the globules were observed within 24h (Fig. S2a and S2b). The $W_1/O/W_2$ emulsion made with sunflower oil was dispersed homogeneously throughout the yogurt and no flocculation or coalescence was observed (Fig. S2c and S2d). Based on these results sunflower oil was used as the dispersed phase in further experiments.

3.3 Effect of $W_1/O/W_2$ emulsion on yogurt formation

The acidification kinetics are essential to yogurt production as prolonged acidification can lead to undesirable physicochemical and sensorial properties (Mishra et al., 2013). Some variation was observed in the acidification profiles $V_{max,}T_{Vmax}$, pH_{Vmax}, and $t_{pH4.6}$ between the yogurt with $W_1/O/W_2$ emulsion and control (Fig.1). A constant pH decrease was recorded in the fermenting milk until the introduction of $W_1/O/W_2$ emulsion (at pH 5.7 ± 0.1, 180min) to the system (Fig.1.). The addition of the $W_1/O/W_2$ emulsion resulted to a slight increase in the pH (~0.2 pH units) while the control, showed a slower acidification profile until 180 min followed by a sharp decline. The differences in the fermenting profiles were verified by the maximum acidification rate, V_{max} of the control yogurt was ~1.6 times higher compared to the yogurt with $W_1/O/W_2$ emulsion ($20.9x10^{-3}$ and $13x10^{-3}$ pH units/min respectively) (Fig 1). The V_{max} was reached earlier in control compared with $W_1/O/W_2$ emulsion (3.5 *vs* 4.5 h, respectively). Despite the fact that adding the $W_1/O/W_2$ emulsion varied the rate of acidification, the overall duration of the process was not affected and was completed by reaching pH 4.5 within 5h in all yogurt samples.

The evolution of *L. bulgaricus* and *S. thermophilus* during the acidification process is shown in Table S1. The addition of $W_1/O/W_2$ emulsion affected the growth pattern of *S. thermophilus* in yogurt which after 3 hours was significantly ($P<0.05$) higher in the control (9.49 ± 0.21 log_{10}CFU/g) while the population remained constant in models with $W_1/O/W_2$ emulsion. Interestingly, *L. bulgaricus* growth did not follow a similar pattern and remained unaffected by the addition of the $W_1/O/W_2$ emulsion.

The physicochemical properties of the fermenting system changed rapidly

during the acidification process. The drop in pH was accompanied by a gradual increase in acidity content in all the yogurts (Fig. 2a.). The accumulation profile of lactic acid was not affected by the addition of $W_1/O/W_2$ and reached a maximum of ~0.75 % w/w by the end of the acidification process at 5h. As it would be expected water retention capacity was increased during the acidification process, sharply at pH 5.7 (3 hours), however, it was significantly less in yogurt samples with $W_1/O/W_2$ emulsion (Fig. 2b.). Initially, syneresis (i.e. the whey separation) was reduced in all yogurt samples and remained constant with no significant difference between all samples throughout the acidification process (Fig. 2c.). The viscosity of pasteurised milk (16%w/v total soluble solids) prior to fermentation was 9.98 mPa.s (data not shown). After 3h of acidification, the viscosity values of all samples were almost doubled marking the onset of the formation of acid induced gel (Fig. 2d.) and remained lower in yogurts with $W_1/O/W_2$ emulsion. The introduction of the $W_1/O/W_2$ emulsion to the fermenting system at 3h (pH~5.7) led to a decrease in the apparent viscosity after 4h. Overall, the addition of $W_1/O/W_2$ emulsion during the acidification process affected the microbial population evolution and formation of the yogurt system.

3.4 Effect of $W_1/O/W_2$ emulsion on the stability of yogurt during storage

3.4.1 Physicochemical properties

Post-acidification physicochemical changes (Fig.3), texture profile (Table1) of yogurt and microbial cell viability (Fig.4) during storage at 4^oC was monitored for 28 days. The pH evolution and lactic acid concentration during storage are considered key criteria of acidity and acceptability of yogurts (Tamime et al., 2007). During the first 6 days, the pH was lower in yogurts without $W_1/O/W_2$

emulsion (Fig. 3a). The acidity profiles followed a similar pattern in all samples and maximum (1.4 % w/w) was achieved after 12 days of storage with changes in pH. At 14 days, a decrease in the acidity content was accompanied by pH increase suggesting that the presence of the $W_1/O/W_2$ emulsion did not affect the pH values and the acidity content of the yogurt system during storage.

The water retention capacity followed a similar pattern in all samples within the first 14 days of storage (Fig. 3b.). On day 21 onwards, the control maintained a significant ($P<0.05$) decrease in water retention capacity which was not evident in samples containing $W_1/O/W_2$ emulsion. Syneresis remained constant during the 21 days of storage for yogurts containing the $W_1/O/W_2$ emulsion (Fig. 3c.). Although syneresis was significantly higher ($P<0.05$) in yogurt containing $W_1/O/W_2$ emulsion at the end of the storage period, all the yogurt samples followed a similar trend. A pronounced difference in the viscosity data between samples containing $W_1/O/W_2$ emulsion and the control was evidenced during the first 6 days of storage (Fig. 3d.). However, these differences were reduced after 6 days and after 28 days, control yogurt was less viscous than the one with $W_1/O/W_2$ emulsion. Overall, the yogurt containing the $W_1/O/W_2$ emulsion presented a stable coherent structure throughout the storage period of 28 days.

Hardness, adhesiveness, cohesiveness and gumminess were examined with texture analysis (Table 1). These characteristics are considered important for consumer acceptability. All yogurts exhibited similar hardness at the onset of the storage period, however, samples containing $W_1/O/W_2$ emulsion seemed

to be harder (i.e. more firm) than the controls during the first two weeks of storage and equilibrated on day 21. Yogurts with $W_1/O/W_2$ emulsion exhibited very different adhesiveness, cohesiveness and gumminess profiles compared to controls. Control yogurts were less adhesive and more cohesive throughout the storage period. Fluctuations were recorded for both samples. Cohesiveness increased in both cases, reaching its maximum at 14 days of storage. Overall, yogurts containing the $W_1/O/W_2$ emulsion exhibited higher values of gumminess (i.e. required more energy to disintegrate), however, this trend was reversed at the end of storage.

3.4.2 Bacteria viability

The population evolution of *L. bulgaricus* and *S. thermophilus* present in the yogurt culture during storage is shown in Fig. 4. *S. thermophilus* population exhibited high viability throughout the storage period (>8 $\log_{10}$CFU/g yogurt) which occasionally was lower in samples with $W_1/O/W_2$ emulsion. A decrease in the viability (~1 log) of the *S. thermophilus* was observed in the control yogurt at the end of the storage period which was not evident in yogurt with $W_1/O/W_2$ emulsion. *L. bulgaricus* population exhibited similar viability profile during storage, and on day 28 a sharp decrease in the population was recorded for both control (~4 log) and yogurt containing $W_1/O/W_2$ emulsion (~2 log), remaining higher in samples with $W_1/O/W_2$ emulsion. The results suggest that the $W_1/O/W_2$ emulsion affected the viability of *S. thermophilus* and *L. bulgaricus* populations compared to controls.

3.4.3 Microscopic observation of stability of $W_1/O/W_2$ emulsion during storage

The structure of yogurts was monitored with light microscopy (Fig. S3.) and cryo-SEM during storage (Fig. 5). The $W_1/O/W_2$ emulsion incorporated within the yogurt remained stable throughout the storage period and the oil globules seem to become part of the gel network of the yogurt. The inner W_1 phase of the oil globules was maintained throughout the 28 days of storage. There was no loss of the inner W_1 phase detected during the first 14 days of storage. Although inner W_1 phase was partially lost in some oil globules, the majority retained their inner W_1 phase until the end of the storage period. Furthermore, no flocculation or aggregation between the oil globules was observed over time. These results suggest that $W_1/O/W_2$ emulsion incorporated within the yogurt matrix exhibited prolonged stability under the storage conditions.

Observing the structure of the samples using cryo-SEM confirmed that $W_1/O/W_2$ emulsion was incorporated successfully into the yogurt structure as the oil globules seemed to be part of the gel network (Figs. 5a., 5b.). Moreover, SEM analysis confirmed the observations made using light microscopy that the inner phase was still retained within the oil globules until the end of the storage period (Figs. 5e., 5f.).

4 Discussion

Based on microscopic observation butterfat-based emulsions did not make a stable dispersed phase. Butterfat is partially solid at room temperature forming a three-phase system that consists of water, oil, and crystals. The crystals pierce the interfacial film of the $W_1/O/W_2$ emulsions globules thereby facilitating their coalescence (Frasch-Melnik, Spyropoulos, & Norton, 2010). Such instabilities were not observed with sunflower oil most likely due to its

low freezing point (-20^{o}C) which prevents the formation of crystals.

Differences in the acidification profile of yogurts with and without $W_1/O/W_2$ emulsion were reflected in the corresponding acidification parameters. The V_{max} values recorded in the yogurt with the $W_1/O/W_2$ emulsion were comparable with those reported for yogurts fermented with other starters and cow milk (19.89-23.44 x10^{-3} pH units/min) (Medeiros, Souza, & Correia, 2015). Addition of ingredients in the fermenting system can interfere with the buffering capacity of the milk leading to lower V_{max} values (Do Espírito Santo, Perego, Converti, & Oliveira, 2012). In the case of $W_1/O/W_2$ emulsion, the constituents of unfermented milk in the outer phase can act as such ingredient. Milk constituents (e.g. soluble phosphate, colloidal calcium phosphate, caseins and whey proteins) affect its buffering capacity (Salaün, Mietton & Gaucheron, 2005) and thus when a portion of the W_2 phase (unfermented milk containing the W_1/O emulsion) is introduced to the fermenting system it can alter its acidification kinetics. As expected, the drop in the pH was accompanied by a gradual increase in acidity content. The recorded values were comparable to literature for yogurt fermentation made with cow milk (Kristo *et al*., 2003; do Espírito Santo et al., 2012; Mishra and Mishra 2013; Medeiros *et al*., 2015). Casein micelles start to aggregate at a ~ pH 5.3, which also causes the solubilisation of colloidal calcium phosphate and change in viscosity (Mishra et al., 2013). Compared to controls yogurts in this study, the Tv_{max} was delayed when adding $W_1/O/W_2$ emulsion and $T_{pH4.6}$ was prolonged. Thus, when a portion of the W_2 phase (unfermented milk containing the W_1/O emulsion) is introduced to the fermenting system it can alter its acidification kinetics.

The growth of *S. thermophilus* during acidification was lower in the presence of $W_1/O/W_2$ emulsion. The addition of $W_1/O/W_2$ emulsion to the acidifying milk did not affect the growth of *L. bulgaricus* but may have altered its proteolytic activity. This in turn could affect the growth of *S. thermophilus*which is stimulated by the bioavailability of free amino acids and peptides present within the milk, released due to the proteolytic activity of *L. bulgaricus* (Tamime et al., 2007). The introduction of $W_1/O/W_2$ emulsion could affect the concentration, availability, and diffusion rates of nutrients (Brocklehurst et al., 1995) altering the growth kinetics of bacteria. Brocklehurst et al. (1995) investigated the growth of *Listeria monocytogenes* and *Yersinia enterocolitica* in O/W emulsions made with 30, 70 and 83% hexadecane or sunflower oil as the dispersed phase, and found that bacteria were restricted to grow as colonies in emulsions containing 83% oil and the growth rates were reduced with increasing oil concentration. The authors concluded that in emulsions bacteria had reduced growth rates and formed colonies due to restricted diffusion of nutrients and oxygen or accumulation of waste products of metabolism (Brocklehurst et al., 1995). *Streptococcus thermophilus* growth is known to be stimulated by the bioavailability of free amino acids and peptides present within the milk, released due to the proteolytic activity of *L. bulgaricus* (Tamime et al., 2007). Also, *L. bulgaricus* is stimulated by S*. thermophilus* in symbiotic fermentation (Zourari, Commissaire & Desmazeaud, 1992). Since the growth patterns of *L. bulgaricus* in control yogurt compared to yogurt with $W_1/O/W_2$ emulsion were similar it was indicated that the symbiosis during acidification was not interrupted by the $W_1/O/W_2$ emulsion. Also, the presence of $W_1/O/W_2$ emulsion had no effect on diffusion of nutrients or oxygen and

waste product accumulation probably because the percentage of emulsion incorporated in the yogurt was not large enough (~33%) to cause such effects. At the end of the storage period, the *S. thermophilus* counts were comparable to literature for yogurt fermentation made with cow milk while *L. bulgaricus* counts were lower (Batista et al., 2015). The latter could due to the presence of ingredients such as glucose, strawberry pulp and/or glucose oxidase (Batista et al., 2015).

Changes in viscosity of the control yogurt verified the three-step structure formation process proposed by Parnell-Clunies, Kakuda, DeMan and Cazzola (1988), i.e. an initial lag period of low viscosity followed by a period of rapid increase and a final stage of high viscosity. The introduction of the $W_1/O/W_2$ emulsion to the fermenting system led to a small decrease in the apparent viscosity after 4 hours, indicating a disturbance in the yogurt structure formation. A well-defined 3-D network is formed as the pH drops initiated by caseins that form aggregates at pH<5.2 (Tamime et al., 2007). At the micro-level the strength and number of bonds between the micelles as well as their structure and spatial distribution affect the viscosity of the yogurt (Lucey, 2002). Casein micelles start to aggregate at a pH close to ~5.3, which also causes the solubilisation of colloidal calcium phosphate and the change in viscosity (Mishra et al., 2013). Overall, the presence of $W_1/O/W_2$ emulsion in the yogurt decreased significantly the viscosity compared to yogurt without $W_1/O/W_2$ emulsion. Similar observations were reported by Izadi, Nasirpour, Garoosi and Tamjidi (2015) with the addition of W/O emulsion to yogurt. In this study, the $W_1/O/W_2$ emulsion was mixed with the fermenting milk at pH values close to 5.7, thus the fermented milk is semi-solid and viscosity

prevented creaming of oil globules and there was better dispersion within the yogurt matrix. At such pH values the structure that is forming is still weak and was probably partially disrupted by the presence of the oil globules resulting in a yogurt with a significantly ($P<0.05$) lower viscosity value compared to the control yogurt immediately after the acidification process. During storage, the viscosity of control yogurt was stabilised to follow a similar trend with yogurt containing $W_1/O/W_2$ emulsion until the end of the storage period. Also, cryo-SEM images showed that the $W_1/O/W_2$ emulsion globules maintained their structure within the yogurt at the end of the storage period. The viscosity of yogurt is expected to decrease during storage although there are cases that the rearrangement of protein and protein-protein contact lead to increased values of viscosity (Izadi et al., 2015).

The water retention capacity was affected by the incorporation of $W_1/O/W_2$ emulsion, specifically, it was significantly lower compared to controls during acidification. Most likely the presence of the oil globules partially disrupted the gel formation, altering the ability of the structure to retain water. During storage the water retention capacity of control yogurt was reduced at day 21 and 28 while in yogurt containing $W_1/O/W_2$ emulsion it was stable throughout the storage period. Set-type yogurts are known for loss in the water retention capacity during storage (Sahan, Yasar & Hayaloglu, 2008; Supavititpatana, Wirjantoro & Raviyan, 2010; Tamjidi, Nasirpour & Shahedi, 2012) which seems to be related with amino acid composition, protein conformation and surface polarity/hydrophobicity. There was no significant difference in syneresis values between all samples at the end of the acidification process. Syneresis marks the deterioration of the protein network and the subsequent

loss of the serum phase from the yogurt gel (Lucey, 2002). Despite the fact that the stirring stage during the introduction of the $W_1/O/W_2$ emulsion to the yogurt was expected to stimulate syneresis (Ozturkoglu-Budak, Akal & Yetisemiyen, 2016), however, this was not the case in the present study. In literature, syneresis values seem to be inversely related to fat content, i.e. increased fat content reduces the whey released due to the increased interactions between the fat globules and the protein network (Akgun, Yazici, & Gulec, 2016).

Textural and rheological properties of yogurts are determined to a great extent by their internal structure. Set-type yogurts should be firm but spoonable (Tamime et al., 2007), thus hardness, cohesiveness, adhesiveness and gumminess are considered important for this type of yogurts (Domagala, Sady, Grega, & Bonczar, 2006). Hardness is among the most commonly evaluated characteristic and expresses the force necessary to attain a given deformation. After the acidification process, the hardness values were higher in control yogurt compared to yogurt containing $W_1/O/W_2$ emulsion. The lower hardness values in the yogurt containing the $W_1/O/W_2$ emulsion can be attributed to a looser gel network. The oil globules of the $W_1/O/W_2$ emulsion should disrupt the yogurt structure leading to softer products. However, during storage yogurts with $W_1/O/W_2$ emulsion showed higher hardness. Although adhesiveness was higher in yogurts with $W_1/O/W_2$ emulsion compared to control after the acidification process, yogurts with $W_1/O/W_2$ emulsion showed lower adhesiveness values during storage which could be attributed to the presence of the oil since higher adhesive values have been recorded for yogurts with low fat content (Domagala et al., 2006).

There was no difference in trend of cohesiveness values between control yogurt and yogurt containing $W_1/O/W_2$ emulsion. Higher fat levels are associated with higher cohesiveness values (Akgun et al, 2016), however, in this study the amount of oil added to the system after incorporating $W_1/O/W_2$ emulsion during the acidification process is probably too low (~6.6%) to cause changes in cohesiveness. During storage, cohesiveness levels remained rather stable for both yogurt samples, coinciding with the data from Akgun et al. (2016). After the acidification process yogurt containing $W_1/O/W_2$ emulsion had lower gumminess values which can be attributed to the oil phase of the emulsion (Kumar & Mishra, 2003). Further work to include sensory (Fonseca et al., 2016) and descriptive analysis with consumers (Torres et al., 2017) would be required to better evaluate the quality and acceptability of the set-style yogurt containing $W_1/O/W_2$ emulsion.

5 Conclusions

The addition of $W_1/O/W_2$ emulsion and its even distribution in a yogurt model was possible resulting in a product with comparable physicochemical characteristics to control yogurt, stability in texture, and retrained high bacterial survival throughout the storage period. The incorporation of $W_1/O/W_2$ emulsion in yogurt structure caused no major alterations in the values of key textural properties. However, consumer tests need to be carried out in order to clarify any variation in perception. Probiotics or nutrients sensitive to manufacturing and storage conditions of yogurt could be encapsulated in the inner W_1 phase avoiding interference with fermentation.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Akgun, A., Yazici, F., & Gulec, H. A. (2016). Effect of reduced fat content on the physicochemical and microbiological properties of buffalo milk yoghurt. LWT- Food Science and Technology, 74, 521–527.

Batista, A. L. D., Silva, R., Cappato, L. P., Almada., Garcia, R. K. A., Silva, M. C., Raices, R. S. L. and Arellano, G. C. (2015). Quality parameters of probiotic yogurt added to glucose oxidase compared to commercial products through microbiological, physical–chemical and metabolic activity analyses. Food Research International, 77, 627–635.

Boitard, L., Cottinet, D., Kleinschmitt, C., Bremond, N., Baudry, J., Yvert, G., & Bibette, J. (2012). Monitoring single-cell bioenergetics via the coarsening of emulsion droplets. Proceeding of the National Academy of Sciences of the United States of America, 109, 7181-6.

Brocklehurst, T. F., Parker, M. L., Gunning, P. A., Coleman, H. P., & Robins, M. M. (1995). Growth of food-borne pathogenic bacteria in oil-in-water emulsions: II-Effect of emulsion structure on growth parameters and form of growth. Journal of Applied Bacteriology, 78, 609–615.

Chen, M., Li, Y., Sun, Q., Pan, A., Manson, J. E., Rexrode, K. M., Willett, W. C., Rimm, E. B. and Hu, F. B. (2016). Dairy fat and risk of cardiovascular disease in 3 cohorts of US adults[1–3]. *The* American Journal of Clinical Nutrition*,* 104, 1209–17.

Do Espírito Santo, A. P., Perego, P., Converti, A., & Oliveira, M. N. (2012). Influence of milk type and addition of passion fruit peel powder on fermentation kinetics, texture profile and bacterial viability in probiotic yoghurts. LWT- Food Science and Technology, 47, 393–399.

Dorobantu, L. S., Yeung, A. K., Foght, J. M., & Gray, M. R. (2004). Stabilization of oil-water emulsions by hydrophobic bacteria. Applied and Environmental Microbiology, 70, 6333-6336.

Domagala, J., Sady, M., Grega, T., & Bonczar, G. (2006). The Influence of storage time on rheological properties and texture of yoghurts with the addition of oat-maltodextrin as the fat substitute. International Journal of Food Properies, 9, 1–10.

EC 1999. Council of the European Union, Council Regulation (EC) No. 1255/1999 on the common organisation of the market in milk and milk products. Published: 2000-05-26

El Kadri, H., Overton, T. W., Bakalis, S., & Gkatzionis, K. (2015).

Understanding and controlling the release mechanism of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules in the presence of NaCl in the W_2 phase. RSC Advances, 5, 105098–105110.

El Kadri, H., Gun, R., Overton, T. W., Bakalis, S., & Gkatzionis, K. (2016). Modulating the release of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure. RSC Advances, 6, 93694–93706.

Farmani, J., Edalatkhah, M., Motamedzadegan, A., & Mardani, M. (2016), Production of set yoghurt analogue through replacement of milk fat with canola and sesame oil. International Journal of Dairy Technology, 69, 433–440.

Firoozmand, H., & Rousseau, D. (2014). Tailoring the morphology and rheology of phase-separated biopolymer gels using microbial cells as structure modifiers. Food Hydrocolloids, 42, 204–214.

Frasch-Melnik, S., Spyropoulos, F., & Norton, I. T. (2010). $W_1/O/W_2$ double emulsions stabilised by fat crystals - formulation, stability and salt release. Journal of Colloid Interface Science, 350, 178–185.

Fonseca, F. G. A., Exmerino, E. A., Filho, E. R. T., Ferraz, J, P., da Cruz, F. G. and Bolini, H. M. A. (2016). Novel and successful free comments method for sensory characterization of chocolate ice cream: A comparative study

between pivot profile and comment analysis. Journal of Dairy Science. 99, 3408–3420.

Hernandez-Marín, N. Y., Lobato-Calleros, C., Roman-Guerrero, A., Alvarez-Ramirez, J., & Vernon-Carter, E. J. (2016). Physical properties and release behaviour of caffeine multiple emulsions stabilised by binary or ternary biopolymer soluble complexes under acid, bile and yogurt storage conditions. Food Hydrocolloids, 58, 42–48.

Izadi, Z., Nasirpour, A., Garoosi, G. A., & Tamjidi, F. (2015). Rheological and physical properties of yogurt enriched with phytosterol during storage. Journal of Food Science and Technology, 52, 5341–5346.

Krasaekoopt, W., Bhandari, B. and Deeth, H. C. (2006). Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage LWT - Food Science and Technology, 39 (2), 177-183.

Kristo, E., Biliaderis, C. G., & Tzanetakis, N. (2003). Modelling of rheological, microbiological and acidification properties of a fermented milk product containing a probiotic strain of *Lactobacillus paracasei*. International Dairy Journal, 13 (7), 517–528.

Kumar, P., & Mishra N. H. (2003). Effect of mango pulp and soymilk fortification on the texture profile of set yoghurt made from buffalo milk. Journal of Texture Studies, 34, 249–269.

Lazaridou, A., Serafeimidou, A., Biliaderis, C. G., Moschakis, T., & Tzanetakis, N. (2014). Structure development and acidification kinetics in fermented milk containing oat β-glucan, a yogurt culture and a probiotic strain. Food Hydrocolloids, 39, 204–214.

Li, B., Jiang, Y., Liu, F., Chai, Z., Li, Y., Li, Y., & Leng, X. (2012). Synergistic effects of whey protein-polysaccharide complexes on the controlled release of lipid-soluble and water-soluble vitamins in W1/O/W2 double emulsion systems. International Journal of Food Science and Technology, 47, 248–254.

Lobato-Calleros, C., Rodriguez, E., Sandoval-Castilla, O., Vernon-Carter, E. J., & Alvarez-Ramirez, J. (2006). Reduced-fat white fresh cheese-like products obtained from $W_1/O/W_2$ multiple emulsions: Viscoelastic and high-resolution image analyses. Food Research International, 39, 678–685.

Lobato-Calleros, C., Sosa-Pérez, A., Rodríguez-Tafoya, J., Sandoval-Castilla, O., Pérez-Alonso, C., & Vernon-Carter, E. J. (2008). Structural and textural characteristics of reduced-fat cheese-like products made from $W_1/O/W_2$ emulsions and skim milk. LWT - Food Science and Technology, 41, 1847–1856.

Lucey, J. A. (2002). Formation and physical properties of milk protein gels. Journal of Dairy Sciences, 85, 281–294.

Ly, M. H., Naïtali-Bouchez, M., Meylheuc, T., Bellon-Fontaine, M. N., Le, T. M., Belin, J. M., & Wache, Y. (2006). Importance of bacterial surface properties to control the stability of emulsions. International Journal of Food Microbiology, 112, 26–34.

Medeiros, A. C., Souza, D., & Correia, R. T. P. (2015). Effect of incubation temperature, heat treatment and milk source on the yoghurt kinetic acidification. International Food Research Journal, 22, 1030–1036.

Mezzenga, R., Folmer, B. M., & Hughes, E. (2004). Design of double emulsions by osmotic pressure tailoring. Langmuir, 20, 3574–3582.

Miles, A. A., Misra, S. S., & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. Journal of Hygiene, 38, 732–749.

Mishra, S., & Mishra, H. N. (2013). Effect of synbiotic interaction of fructooligosaccharide and probiotics on the acidification profile, textural and rheological characteristics of fermented soy milk. Food Bioprocess Technology, 6, 3166–3176.

Muschiolik, G., & Dickinson, E. (2017). Double emulsions relevant to food systems: preparation, stability, and applications. Comprehensive Reviews in Food Science and Food Safety, 16, 532–555.

Ozturkoglu-Budak, S., Akal, C., & Yetisemiyen, A. (2016). Effect of dried nut fortification on functional, physicochemical, textural, and microbiological properties of yogurt. Journal of Dairy Sciences, 99, 8511–8523.

Parker, M. L., Brocklehurst, T. F., Gunning, P. A., Coleman, H. P., & Robins, M. M. (1995). Growth of food-borne pathogenic bacteria in oil-in-water emulsions: I- Methods for investigating the form of growth. Journal of Applied Bacteriology, 78, 601–608.

Parnell-Clunies, E., Kakuda, Y., DeMan, J. M., & Cazzola, F. (1988). Gelation profiles of yogurt as affected by heat treatment of milk. Journal of Dairy Sciences, 71, 582–588.

Patel, A. R., Drost, E., Seijen ten Hoorn, J., & Velikov, K. P. (2013). Fabrication and characterization of emulsions with pH responsive switchable behavior. Soft Matter, 9, 6747–6751.

Pimentel-González, D. J., Campos-Montiel, R. G., Lobato-Calleros, C.,

Pedroza-Islas, R., & Vernon-Carter, E. J. (2009). Encapsulation of *Lactobacillus rhamnosus* in double emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions. Food Research International, 42, 292–297.

Ryeo-Eun, G., Kyung-A, H., Ye-Seul, K., Seung-Hee, K., Ki-Hoan, N. and Kyung-Chul, C. (2015). Effects of palm and sunflower oils on serum cholesterol and fatty liver in rats. Journal of Medicinal Food, 18 (3): 363-369.

Sahan, N., Yasar, K., & Hayaloglu, A. A. (2008). Physical, chemical and flavour quality of non-fat yogurt as affected by a β-glucan hydrocolloidal composite during storage. Food Hydrocolloids, 22, 1291–1297.

Salaün, F., Mietton, B., & Gaucheron, F. (2005). Buffering capacity of dairy products. International Dairy Journal, 15, 95–109.

Shima, M., Morita, Y., Yamashita, M., & Adachi, S. (2006). Protection of *Lactobacillus acidophilus* from the low pH of a model gastric juice by incorporation in a W/O/W emulsion. Food Hydrocolloids, 20, 1164–1169.

Shima, M., Matsuo, T., Yamashita, M., & Adachi, S. (2009). Protection of *Lactobacillus acidophilus* from bile salts in a model intestinal juice by incorporation into the inner-water phase of a W/O/W emulsion. Food Hydrocolloids, 23, 281–285.

Sultana, K. Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P. and Kailasapathy, K. (2000) Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt International Journal of Food Microbiology. 62 (1–2), 47-55.

Supavititpatana, P., Wirjantoro, T. I., & Raviyan, P. (2010). Characteristics and shelf-life of corn milk yogurt. Chiang Mai University Journal of Natural Sciences, 9, 133–150.

Tamime, A., & Robinson, R. (2007). Tamime and Robinson's Yoghurt Science and Technology. (3rd ed.). London: CRC Press.

Tamjidi, F., Nasirpour, A., & Shahedi, M. (2012). Rheological characteristics of yogurt enriched with microencapsulated fish oil. Journal of Agricultural Science and Technology, 16, 1073–1082.

Torres, F. R., Esmerino, E. A., Carr, B. T., Ferrão, L. L., Granato, T., Pimentel, T. Bolini, H. M. A., Freitas, M. Q. and Cruz, A. G. (2017). Rapid consumer-based sensory characterization of requeijão cremoso, a spreadable processed cheese: Performance of new statistical approaches to evaluate check-all-that-apply data. Journal of Dairy Science. 100, 6100–6110.

Weiss, J., & Muschiolik, G. (2007). Factors affecting the droplet size of

water-in-oil emulsions (w/o) and the oil globule size in water-in-oil-in-water emulsions (w/o/w). Journal of Dispersion Science and Technology, 28, 703–716.

Zourari, A., Commissaire, J., & Desmazeaud, M. J. (1992). SDS-solubilized whole-cell protein patterns of *Streptococcus salivarius* subsp. thermophilus and *Lactobacillus delbrueckii* subsp. bulgaricus isolated from Greek yogurts. Journal of Dairy Research, 59, 102–109.

Captions

Fig. 1. Acidification profiles of milk fermented with yogurt starter culture with (squares) and without (circles) $W_1/O/W_2$ emulsion. Error bars represent the standard deviation (sd) of the mean value (*n*=3).

* Time point of $W_1/O/W_2$ emulsion addition (180min) is represented by two pH measurements before and after addition.

As inset table the corresponding acidification parameters: acidification rate (Vmax), time to reached Vmax (h), t_{Vmax}; pH at Vmax, pH_{Vmax}; and the time to complete the fermentation (h), $t_{pH4.6}$. Mean value of three independent experiments ± sd; Mean values in the same column with the same superscript indicate no significant differences ($p < 0.05$).

Fig. 2. Kinetics of (a) titratable acidity, (b) water retention capacity, (c) syneresis (d) viscosity, during the acidification process of milk with $W_1/O/W_2$ emulsion and control. Error bars represent the standard deviation (sd) of the mean value (*n*=3).

Fig. 3. Kinetics of (a) ph values and titratable acidity, (b) water retention capacity (c) syneresis and (d) viscosity during storage of yogurt with (bars with vertical stripes) and without (plain bars) the addition of $W_1/O/W_2$ emulsion. Error bars represent the standard deviation (sd) of the mean value (*n*=6).

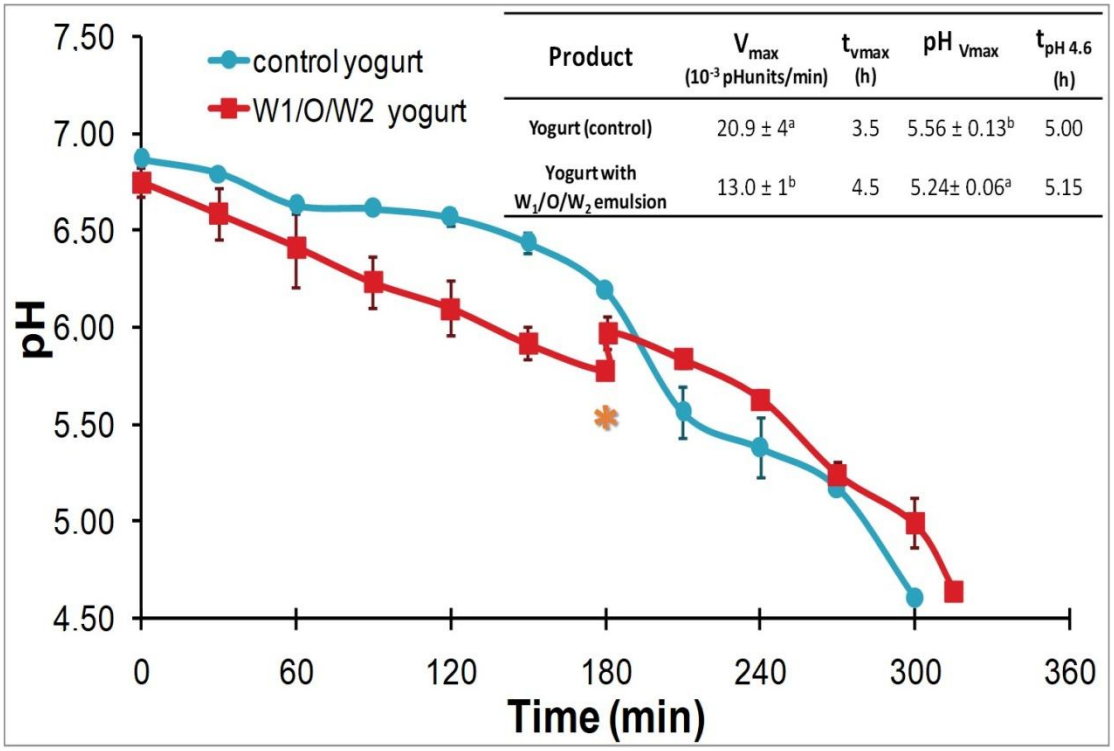
Fig. 4. Cell viability of (a*) L. bulgaricus* and (b) *S. thermophillus* during storage of yogurt with $W_1/O/W_2$ emulsion (striped bars) and control (plain bars). Error bars represent the standard deviation (sd) of the mean value (*n*=6).

Fig. 5. Scanning electron microscope (SEM) image of the cryo-fractured yogurt samples containing $W_1/O/W_2$ emulsion (a) at the beginning of the storage period. (0 days) Scale bar, 200 μm. (b) Zoomed SEM image of a. Scale bar, 10 μm (c) after 2 weeks of storage at 4 ^{o}C. Scale bar, 100 μm (d) Zoomed SEM image of b Scale bar, 20 μm and (e) at the end of the storage (after 4 weeks) at 4 ^{o}C. (f) Zoomed SEM image of e. Scale bar, 10 μm.

Table 1. Effect of W1/O/W2 emulsion on the texture profile of yogurts during storage.

Mean value of six independent measurements ± standard deviation (sd);
Mean values in the same row with the same superscript indicate that there are no significant differences between them ($P<0.05$)

Figure 1



Product	V_{max} (10^{-3} pHunits/min)	t_{vmax} (h)	pH $_{Vmax}$	$t_{pH\ 4.6}$ (h)
Yogurt (control)	20.9 ± 4[a]	3.5	5.56 ± 0.13[b]	5.00
Yogurt with $W_1/O/W_2$ emulsion	13.0 ± 1[b]	4.5	5.24± 0.06[a]	5.15

Figure 2a

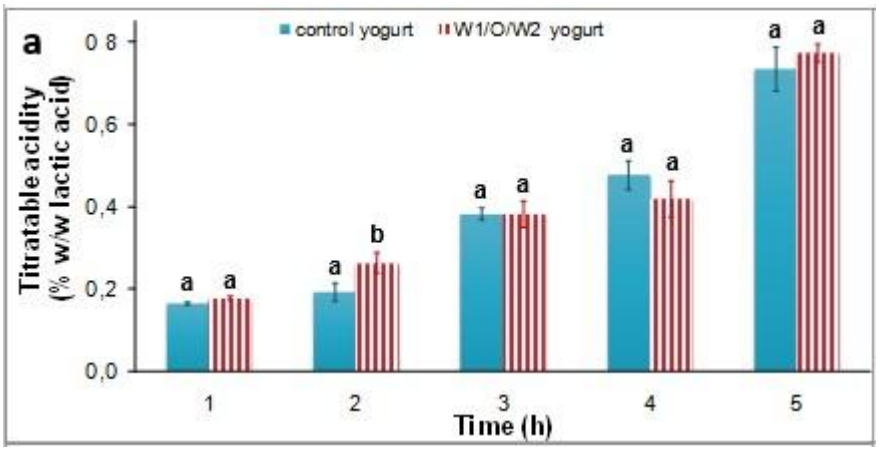


Figure 2b

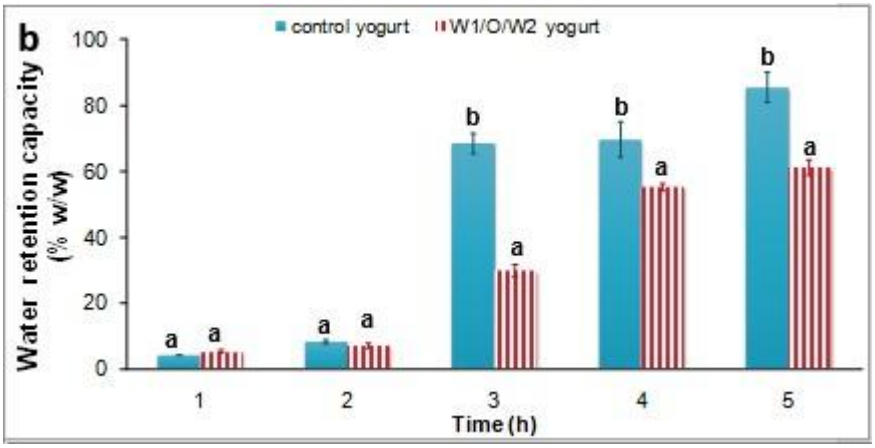


Figure 2c

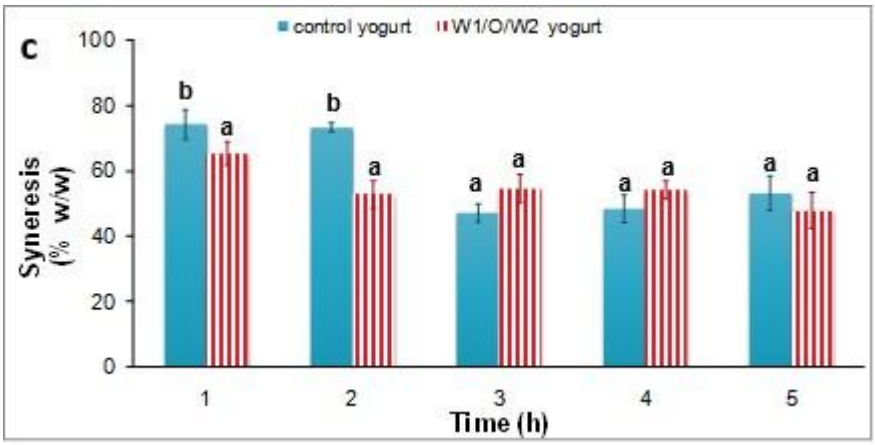


Figure 2d

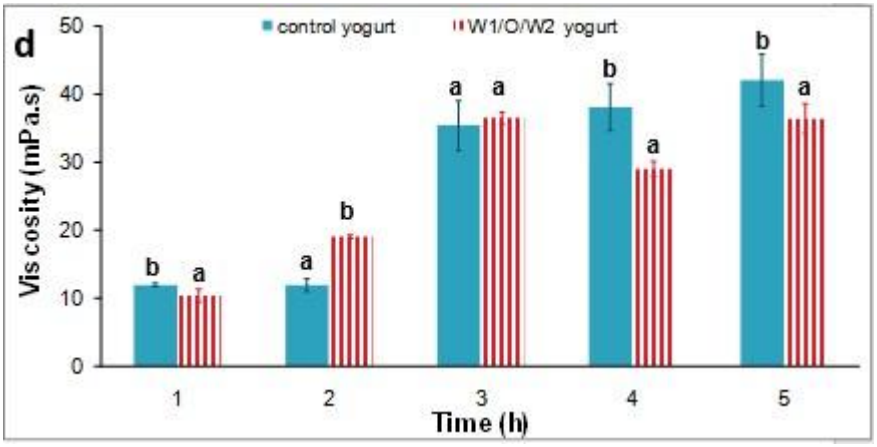


Figure 3a-b

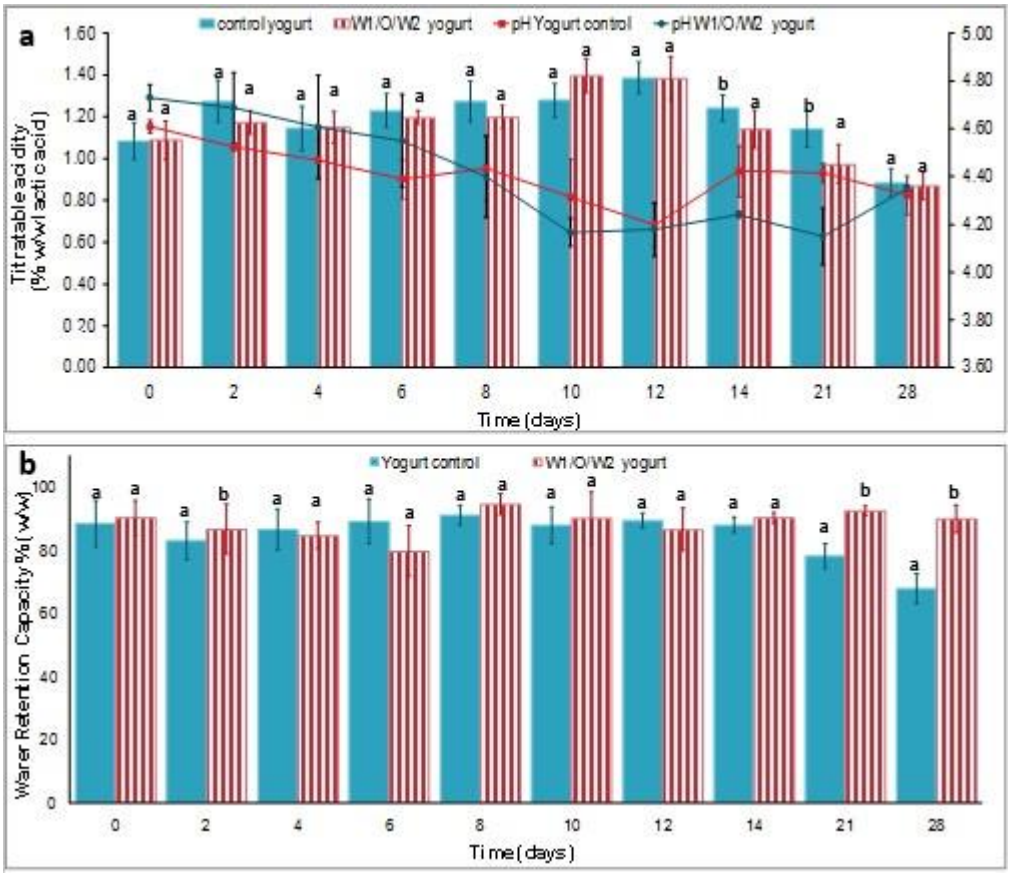


Figure 3c-d

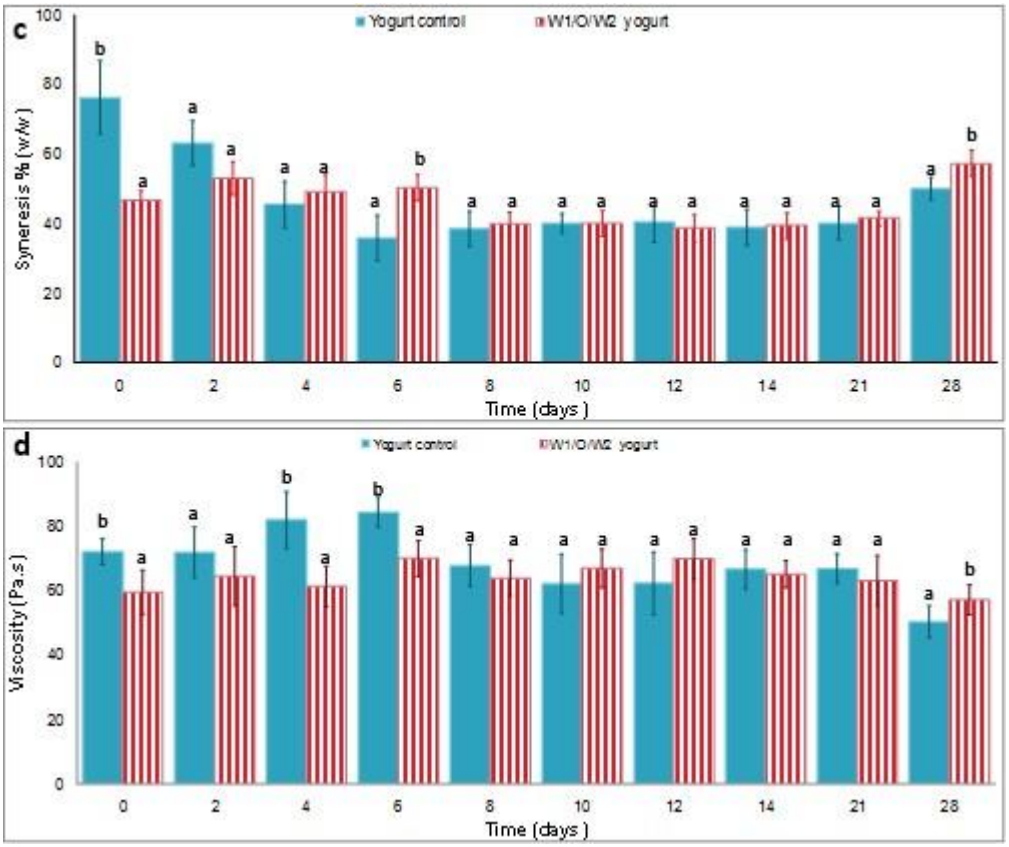


Figure 4

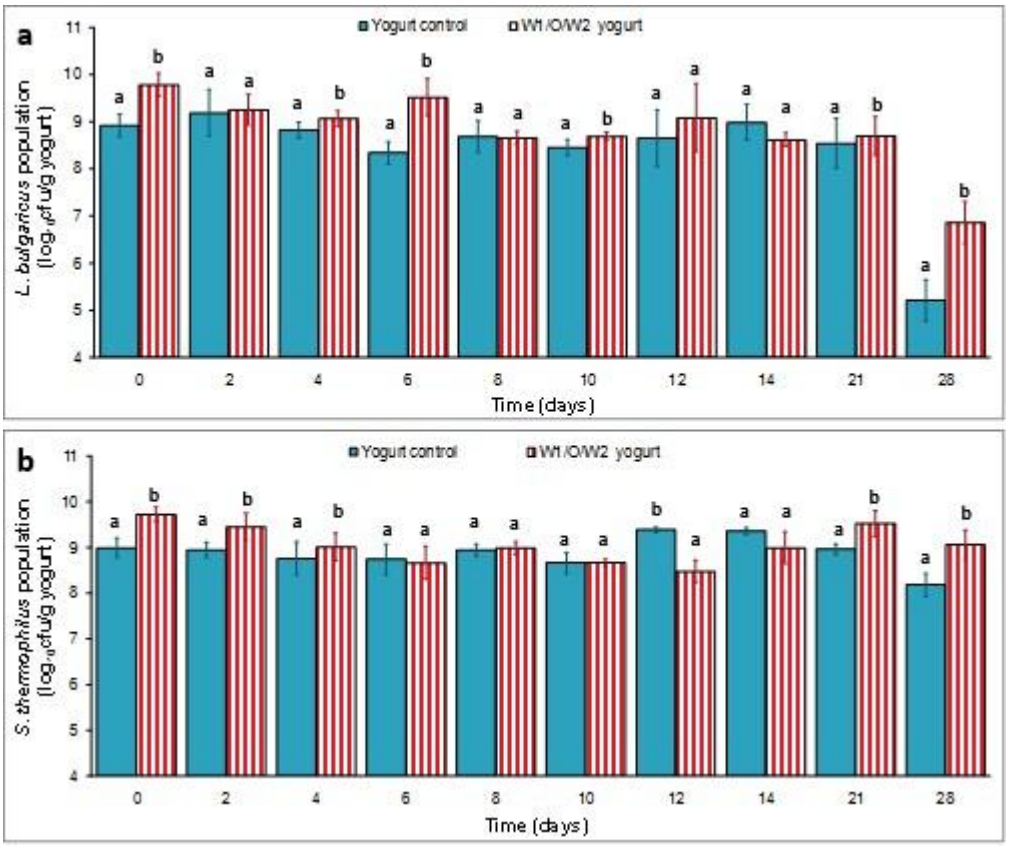


Figure 5

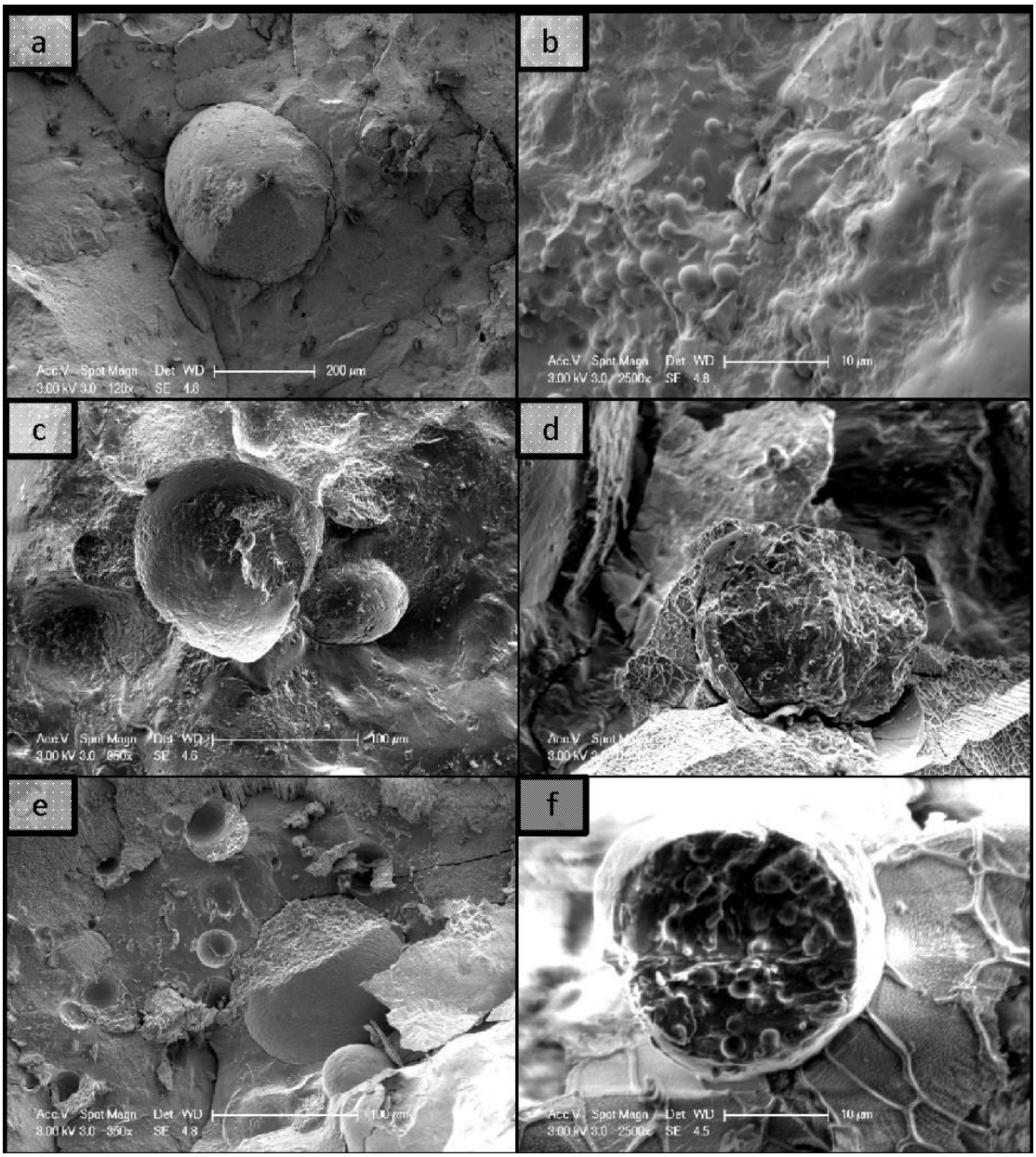


Table 1. Effect of W1/O/W2 emulsion on the texture profile of yogurts during storage.

Variables	Days	Yogurt samples	
		Yogurt control	Yogurt $W_1/O/W_2$ emulsion
Hardness	0	79.23 ± 12.91 [a]	67.43 ± 7.73 [a]
	2	67.35 ± 6.27 [a]	83.07 ± 8.66 [b]
	4	68.67 ± 7.75 [a]	96.62 ± 6.79 [b]
	6	69.40 ± 10.78 [a]	90.08 ± 12.89[b]
	8	61.15 ± 7.97 [a]	87.47 ± 14.76[b]
	10	56.27 ± 3.44 [a]	63.54 ± 5.52 [b]
	12	49.90 ± 6.50 [a]	67.30 ± 4.18 [b]
	14	48.43 ± 4.78 [a]	72.08 ± 7.45 [b]
	21	89.12 ± 12.67 [a]	92.74 ± 7.58 [a]
	28	121.97 ± 17.56 [a]	97.00 ± 10.84 [a]
Adhesiveness	0	-150.52 ± 16.09 [a]	-88.70 ± 7.56 [b]
	2	-46.39 ± 4.30 [b]	-169.62 ± 23.46 [a]
	4	-87.48 ± 10.97 [b]	-132.60 ± 22.94 [a]
	6	-97.30 ± 7.50 [b]	-115.61 ± 15.28 [a]
	8	-46.85 ± 6.57 [b]	-134.97 ± 22.96 [a]
	10	-39.71 ± 4.68 [b]	-67.41 ± 10.06 [a]
	12	-43.79 ± 5.30 [b]	-53.99 ± 7.57 [a]
	14	-20.29 ± 1.58 [b]	-80.08 ± 7.35 [a]
	21	-53.73 ± 7.93 [b]	-100.84 ± 9.91 [a]
	28	-208.01 ± 26.68 [b]	-114.95 ± 11.83 [a]
Cohesiveness	0	0,82 ± 0,04 [b]	0,72 ± 0,04 [a]
	2	0,87 ± 0,09 [b]	0,74 ± 0,05 [a]
	4	0,83 ± 0,04 [a]	0,83 ± 0,05 [a]
	6	0,87 ± 0,06 [b]	0,79 ± 0,03 [a]

	8	0,88 ± 0,05 [b]	0,76 ± 0,05 [a]
	10	0,86 ± 0,05 [a]	0,83 ± 0,02 [a]
	12	0,88 ± 0,05 [b]	0,82 ± 0,03 [a]
	14	0,90 ± 0,04 [b]	0,81 ± 0,05 [a]
	21	0,85 ± 0,07 [a]	0,79 ± 0,04 [a]
	28	0,82 ± 0,09 [b]	0,73 ± 0,03 [a]
Gumminess	0	64,67 ± 10,03 [b]	48,54 ± 6,23 [a]
	2	58,73 ± 7,50 [a]	60,86 ± 4,88 [a]
	4	56,93 ± 5,82 [a]	80,05 ± 8,24 [b]
	6	59,98 ± 7,99 [a]	72,65 ± 7,62 [b]
	8	53,80 ± 8,39 [a]	68,17 ± 5,69 [b]
	10	48,53 ± 2,62 [a]	52,77 ± 3,62 [b]
	12	43,55 ± 5,09 [a]	54,38 ± 3,38 [b]
	14	43,67 ± 4,07 [a]	57,78 ± 4,46 [b]
	21	75,60 ± 9,87 [a]	74,72 ± 5,92 [a]
	28	100,74 ± 19,94 [b]	71,85 ± 7,00 [a]

Mean value of six independent measurements ± standard deviation (sd); Mean values in the same row with the same superscript indicate that there are no significant differences between them ($P<0.05$)

Highlights

- First incorporation of $W_1/O/W_2$ emulsion during set-type yogurt acidification.
- The system was proved stable during acidification and storage.
- Microbial populations were affected by the presence of $W_1/O/W_2$.
- Milk stabilised the O-W2 interface without the need for nonionic surfactant.